BLOOD-BRAIN-BARRIER PENETRATING TGFβ TRAPS FOR TREATMENT OF GLIOBLASTOMA IN COMBINATION WITH IMMUNE CHECKPOINT INHIBITORS

by

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Abstract

Gliomas are primary malignant tumors in the CNS. Glioblastoma is the most common and aggressive form of glioma. One of the reasons that glioblastoma is so difficult to treat is its location. The blood-brain-barrier (BBB) constructs a formidable obstacle to any potential treatment for brain-associated diseases. Transferrin receptor 1 (TfR1) is a receptor expressed on BBB endothelial cells. As TfR1 has been identified as cell surface receptor for human ferritin H chain (FTH), FTH nanocage is an attractive platform for receptor-mediated drug delivery. Recently, improved understanding of immune regulation has led to development in the field of immunotherapy studies to treat glioblastoma. Among them, immune checkpoint signaling inhibition has established a leading role among the immunomodulatory therapies. Brain tumor associated immune suppression is considered to be mediated by transforming growth factor β (TGFβ) while deficiency of response to immune checkpoint inhibitors was associated with symbolic TGFβ signaling in fibroblasts.

In order to eliminate the effect of excessive TGFβ production and promote the efficacy of immune checkpoint inhibitors for treatment of glioblastoma, we proposed fusion proteins FTB04~07 with varying sequences and linkers incorporating human FTH as nanocarriers and TGFβRII ectodomain as TGFβ traps in order to traverse the BBB and promote anti-tumor activity in combination with immune checkpoint inhibitors. Plasmid DNA with fusion protein

sequences were designed to be expressed through E. coli system. Expressed inclusion bodies were solubilized with high concentration of urea and refolded. Fusion protein was purified with Ni-NTA and characterized by GPC analysis and DLS.

For *in vitro* studies, results from HEK-293T SBE-luciferase reporter assay confirmed that FTB04 and FTB05 exhibited superior blockade of TGFβ-induced activity compared to FTB06 and FTB07. ELISA binding affinity studies and SMAD phosphorylation studies indicated that fusion proteins can bind to TGFβ and block TGFβ-induced activity through the blockade of SMAD2/3 phosphorylation. Pull-down study with HepG2 cells proved that fusion proteins can bind to TfR1, confirming their potential to traverse the BBB. FTB04 demonstrated the best performance in all validation studies, thus would be the focus in future studies.

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Chapter 1

Introduction

1.1 Glioblastoma

Due to the localization and locally invasive growth, brain tumors are characterized by their high morbidity and mortality. Gliomas are brain tumors thought to derive from neuroglial stem or progenitor cells. Gliomas are the primary malignant tumors in the central nervous system (CNS), representing 75% of malignant primary brain tumors in adults,[1] and are responsible for the majority of deaths caused by primary brain tumors. Malignant primary brain tumors are among the cancers that are most difficult to treat. Gliomas have been traditionally classified as astrocytomas, oligodendrogliomas, mixed oligoastrocytic gliomas and ependymomas based on their histological appearances and morphological similarities to the normal neuroglial cell types in the brain. The most aggressive form of glioma, glioblastoma, accounts for approximately 50% of cases.[2] Glioblastoma is the most prevalent and lethal type of primary brain tumors, with a median survival of 14 months[3] and 5-years overall survival rate only around 5.5%.[1] Currently, standard of care therapeutic interventions for glioblastoma patients includes surgery, temozolomide chemotherapy, radiotherapy and corticosteroids.[4] Conventional treatments provide patients with additional survival time, while a cure has never been achieved. All patients with glioblastoma eventually have disease relapse. Many reasons led to the lack of

improvement in the outcomes of traditional therapies. Location is one of the most important reasons. The aggressive tumor growth in a vital organ as the brain limits the utility of local therapy, while the blood brain barrier (BBB) provides indiscriminate protection for tumor cells. The blood brain barrier is a defensive interface between the CNS and the peripheral blood circulation, protecting the brain from potential neurotoxins. The blood brain barrier is essential for maintaining homeostasis of the CNS. It mainly comprises vascular endothelial cells of the brain capillaries and the pericytes and astrocytes surrounding.[5] While the blood brain barrier provides significant protection of the brain, it also presents a formidable obstacle to any drug delivery systems aiming for the brain, preventing the potential treatment for brain-associated diseases. Over the past decades, significant efforts have been made to the development of active carrier systems with the ability to traverse the blood brain barrier to treat diseases located in the brain, including brain tumors. However, none of these attempts have been successfully passed on to clinical usage.[6]

1.2 Current Studies of Immunotherapy for Glioblastoma

Over the past decade, improved understanding of immune regulation has led to rapid development in the field of innovative immunotherapy studies to treat glioblastoma. The immune environment and the unique mechanisms of immunosuppression[7] in the CNS requires special considerations when developing immunotherapies. The CNS has been reported as an immune privileged system[8-10] a few decades ago. The CNS presents vigorous immunosurveillance and potent immune responses.[11] It has been reported[12] that most antigen presenting cells from the brain are subject to travel to deep cervical lymph nodes where they

can prime lymphocytes. That is, although the brain is an immunologically distinct organ, such immune environment offers sufficient opportunities for potential immunotherapies. Glioblastoma has been recognized as an exemplification for cancer-related immunosuppression. Impaired cellular immunity in patients with glioblastoma has been reported despite metastasis towards extracranial sites are rarely observed. Li, B. et al.[13] reported that glioblastomas presents comparative deficiency of infiltrating T cells compared to other types of tumors. Even for the T cells that avoid deletion produce insufficient pro-inflammatory cytokines and fail to execute their cytotoxic functions. Tumor-intrinsic factors related to glioblastoma involves the induction of signaling pathways that suppresses immune responses, such as TGFβ, IL-10 and prostaglandins. Studies[14-15] also demonstrated that the interactions between glioblastoma cells and immune cells result in suppression of natural killer cell activity, and steer induction of apoptosis in immune cells. The extensive glioblastoma-associated immunosuppression has limited the use of standard conventional therapies with immunosuppressive effects as well as the design and trials of immunotherapy.

Current state of immunotherapy to treat glioblastoma mainly involves glioblastoma vaccines, oncolytic viruses, chimeric antigen receptor T cell therapy and immune checkpoint inhibitors. Research into vaccination approaches exerts efforts to strengthen the adaptive portion of the immune system by inducing active immune surveillance against tumor cells in the brain. More than 20 approaches of glioblastoma vaccination have entered clinical trials and three of them have already reached phase III development.[2] Chimeric antigen receptors (CARs) are composed of antigen recognition domains of antibodies associated with T cell activation

domains. CAR T cell therapy genetically engineered T cells to express CARs, providing the modified T cells with specificity for tumor-associated antigens. The significant advantage of CAR T therapy was the capacity of engineered T cells to recognize antigens other than MHC molecules, which is required for developing adaptive immune responses, yielding more possibility to overcome immunosuppression of tumor microenvironment. A few CAR T cells development have entered clinical trials and proved that engineered T cells are capable of infiltrating glioblastomas and being activated.[16] However, the immunosuppression within the tumor microenvironment is likely to restrict the anti-cancer activity of CAR T therapy[17], calling for combination therapies or other approaches to improve therapeutic efficacy. Immune checkpoint inhibition has been the most important advance in cancer immunotherapy in recent years. It has been demonstrated that T cell immune responses are controlled through a set of proteins called immune checkpoints that protect the body from possible damaging immune responses.[18] Immune checkpoint inhibition involves antibodies that reduce activity of negative regulatory pathways limiting T cell activation. Modulation of immune regulators exerts universal effect on immune responses, not dependent on specific tumor antigens. The most prominent and well studied examples have been antibodies against cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), or its ligand PD-L1. Numerous immune checkpoint inhibitor therapy for patients with glioblastoma have entered clinical trials and three with Nivolumab (anti-PD-1 antibody) have successfully reached phase III.[2] Nivolumab has been the immune checkpoint inhibitor with most advanced clinical development for patients with glioblastoma. It has been reported to yield durable responses and

long-term disease control in patients with recurrent glioblastoma.[19] Other antibodies for immune regulators such as pembrolizumab have also established certain level of success for patients with glioblastoma in earlier stages of clinical trials.[20] Despite the clinical success of antibodies for immune checkpoint proteins, only a small subset of all patients with glioblastoma exhibits mutation affecting the DNA mismatch repair machinery,[21] suggesting that a broader inclusion of cancer immunity is required for developing immunotherapy.

1.3 PD-1/PD-L1 Immune Checkpoint Inhibitors

As the field of cancer immunotherapy has been rapidly developing over the last decade, PD-1 and PD-L1 immune checkpoint signaling inhibition has established a leading role among the immunomodulatory therapies.[22-24] PD-1 was first reported as a protein expressed on the surface of T cells in 1992[25] and was reported to be related with apoptosis. Further studies demonstrated that PD-1 is a negative regulator of immune responses that limits T cell activation and proliferation.[26] The first ligand identified for PD-1 was a molecule analogous to B7-1 and B7-2 and was first named as B7-H1. It was later recognized as the ligand for PD-1 and was thus named as PD-L1. PD-L1 is expressed on a wide range of different cell types including subsets of immune cells, antigen presenting cells and certain types of tumor cells.[27] The ligand has been reported to be expressed in a subset of glioblastomas but the extent of such expression varies, ranging from around 2% to 88%.[28-29] Interaction between PD-L1 and PD-1 in T cells is reported to result in T cell dysfunction, exhaustion, neutralization and interleukin-10 (IL-10) production in unit tumor mass.[30] The PD-1/PD-L1 signaling pathway physiologically control the extent of inflammation at antigen expressing sites to protect normal tissue from being

damaged. Overexpression of PD-L1 in tumors secure tumor cells from cytotoxic T cell mediated cell death. The paucity of cytotoxic T cell function allows tumor cells to become aggressive and secrete pro-inflammatory cytokines.[31] PD-1 interacts with two ligands PD-L1 and PD-L2, but only the binding of PD-1 to PD-L1 has been confirmed to be responsible for tumor immune modulation. Pharmacologically, PD-1/PD-L1 immune checkpoint inhibitors prevent the PD-1/PD-L1 interaction, facilitating T cell immune response to kill the tumor.

PD-1/PD-L1 inhibition has emerged as a clearly beneficial anticancer therapy. Due to its comparatively low toxicity profile compared to other immune checkpoint inhibitors, PD-1/PD-L1 pathway inhibition therapy has been the most advanced developed approach in clinical trials.[30] Immunotherapeutic approaches applying anti-PD-1 antibodies have reached Phase III clinical trials. To date, five PD-1/PD-L1 immune checkpoint inhibitors have been approved by FDA for use in cancer therapeutics, including nivolumab, pembrolizumab, avelumab, durvalumab and atezolizumab. The first four have all been clinically developed for treatment of glioblastoma.[2] Among them nivolumab, a humanized monoclonal IgG4 anti-PD-1 antibody, has been the most well-developed therapeutics for patients with glioblastoma. Its mechanism includes binding to the PD-1 receptor, thus blocking its interaction with both PD-L1 and PD-L2, releasing the PD-1/PD-L1 pathway mediated immune response against tumor cells, thus increasing anti-tumor activity. Combinations of nivolumab and conventional radiotherapy or other immunotherapeutic approaches such as anti-CTLA-4 antibody have shown benefits in overall survival and safety in clinical trials. Another humanized anti-PD-1 antibody, pembrolizumab, which acts in a similar mechanism as nivolumab, has also been widely applied

and shown notable outcomes.

1.4 TGFβ Signaling

Despite the successful induction of durable responses in patients treated with PD-1/PD-L1 immune checkpoint inhibitors for various types of cancer, such robust responses only occurred in a fraction of patients. Response to PD-1/PD-L1 inhibitors treatment was associated with CD8+ T cells and high neoantigen or tumor mutation burden. On the other hand, deficiency of response was associated with symbolic transforming growth factor β (TGFβ) signaling in fibroblasts.[32] Brain tumor associated immune suppression is also considered to be mediated by TGFβ, which was found to present in the serum of brain tumor bearing mice at an elevated level than in that of mice without brain tumors.[33]

The TGFβ superfamily is comprised of over 30 members of secreted factors in mammals.[34] At cellular level, TGFβ proteins are instrumental in fundamental cell processes regulation. TGFβ is a pleiotropic cytokine associated with poor prognosis in many types of tumors. TGFβ presents as a complex role in cancer. TGFβ has a dual role in tumor development, acting as a tumor suppressor through inhibition of growth and promotion of apoptosis in multiple types of cells, but also as a tumor promoter through enhancing tumor cell growth, invasiveness and metastasis, mainly by modulation of the immune system as well as the tumor microenvironment.[35] The tumor suppressive function of TGFβ pathway is traced by the presence of mutations in TGFβ signaling components. Once the tumor suppression has been overcome, TGFβ switches its role to enhance tumor promotion. TGFβ also present an effect on

tumor-host interaction, suppressing inflammation, regulating immune function, promoting angiogenesis and regulating the interaction between stromal fibroblasts and tumor cells.[36] TGFβ1, a polypeptide comprised of 390 amino acids, is the most abundant and well-studied isoform of TGFβ proteins. TGFβ binding to the type II receptor (TGFβR2) results in the activation of type I receptor (TGFβR1) to yield a TGFβ binding complex of high specificity[37], thus initiates cytoplasmic signaling pathways to produce cellular responses.

The linkage between cell-surface transmembrane receptors and target genes in TGFβ signaling pathway is a type of small intracellular effector proteins known as Smads.[38] It has been reported[39] that Smads are phosphorylated and accumulated in the nucleus in response to TGFβ agonists. Smads are categorized into three different classes: the receptor -regulated Smads (R-Smads), which can be divided into bone morphogenetic proteins (BMP)-activated Smads and TGFβ-activated Smads, the common mediator Samd (Co-Smad) and the inhibitory Smads. TGFβ-activated R-Smads include Smad2 and Smad3 and serve as substrates of TGFβ receptors. Genetic studies[40] have identified a clear and pivotal role of Smad-dependent TGFβ signaling pathway in tumor suppression in a variety of human cancer types.

There are three common therapeutic strategies against TGFβ signaling[41]: at the ligand level, antisense molecules are used to prevent TGFβ synthesis; at the ligand-receptor level, ligand traps and anti-receptor monoclonal antibodies are applied to prevent ligand-receptor interactions; at intracellular level, receptor kinase inhibitors and peptide aptamers are adopted to prevent signal transduction. In the tumor microenvironment, ligand traps can control the

excess of TGFβ production. Ligands traps include TGFβ receptor antibodies, neutralizing TGFβ antibodies and soluble TGFβ receptors. Therapeutic approaches applying soluble TGFβR2 and betaglycan have been developed tested in pre-clinical studies. It has been proved[32,41] that co-administration of anti-TGFβ antibodies and anti-PD-L1 antibodies facilitated T cell infiltration into the center of tumors, provoked vigorous anti-tumor immunity and tumor regression.

1.5 Ferritin Nanocarrier to Overcome BBB

Ferritin is a spherical molecule composed of 24 subunits of a mixture of ferritin H chain (FTH) and ferritin L chain (FTL). FTH and FLH are named for their initial isolation from heart and liver, respectively. Molecular weight of human FTH is around 21 kDa while that of human FTL is around 19 kDa, so these subunits are also referred to as ferritin heavy chain and ferritin light chain. Ferritin exerts its function of storage of iron within cells while it also circulates and binds specifically to multiple cell types in a saturable manner. Previous studies[42] found out that binding and uptake of human ferritin are mediated by transferrin receptor 1 (TfR1). TfR1 was identified as a significant endocytosing receptor for FTH on cell surface but possesses little or no binding to FTL. These studies also indicated that binding of FTH to TfR1 facilitated entry of FTH into endosomes and lysosomes.[43] Therefore, receptor-mediated transport (RMT) utilizing FTH as a carrier has been identified as one attractive platform for drug delivery.

Highly expressed RMT system receptors in blood brain barrier endothelial cells mostly involve three different receptors: TfR1, insulin receptors and low-density lipoprotein receptors.[44] TfR1

has been widely studied to be over-expressed in many tumors including brain tumor tissues.[45] Human TfR1 was proved to be highly expressed in the BBB and anti-TfR1 antibodies significantly blocked the binding of FTH to human BBB endothelial cells. Thus, it also demonstrated that FTH can overcome the BBB through receptor-mediated transport. *Fan et al.* reported[46] successful BBB transcytosis both *in vitro* and *in vivo*. In the same study for glioma treatment, *in vivo* imaging illustrated that after traversing the BBB, the amount of accumulated FTH nanocarriers in the brain in the tumor area was 10-fold higher in comparison with that in adjacent healthy brain tissue. Results from previous studies indicated that FTH nanocarriers possess the ability to traverse the BBB *via* receptor-mediated transport and target and accumulate in glioma disease sites specifically.

1.6 Fusion Protein

Based on the results from previous studies, we designed fusion proteins incorporating human ferritin heavy chain as nanocarriers and TGFβ type II receptor ectodomain as TGFβ traps in order to traverse the blood brain barrier and promote anti-tumor activity in combination with PD-1/PD-L1 immune checkpoint inhibition therapies. Protein nanocage consisted of 24 subunits of human ferritin heavy chain has been reported as a promising drug delivery system, specifically targeting transferrin receptor 1. TGFβ receptor 2 is composed of a C-terminal protein kinase domain and an N-terminal ectodomain. Among them, the ectodomain serves as the extracellular ligand-binding domain with TGFβ.[47] Here, we utilize the potential of the human FTH nanocages to traverse human BBB and target glioma cells as well as the cage structure of the protein to maximum interaction between TGFβ and TGFβ traps for higher level

of TGFβ signaling pathway blockade. In order to facilitate protein preparation and *in vitro* assessments, a 6x poly-histidine tag (His-tag) was fused to the protein sequence. His-tag is commonly used for protein affinity purification and binding assessments due to its binding affinity with commercial carriers such as Ni-NTA and some other metal ions including copper and cobalt. To provide the desired protein structure and satisfactory ligand-receptor interaction, two types of linkers were included in the sequence between the ferritin heavy chain subunit and the TGFβR2 ectodomain. A flexible GS-linker was fused into FTH-TGFβRIIecto fusion protein 04 (FTB04) and a rigid linker was involved in FTB05. Switched positions of FTH1 and TGFβRII ectodomain provided FTB06 and FTB07 with different linkers, respectively. These four types of recombinant fusion proteins were designed to be plasmid DNA vectors to express in *E. coli* bacterial system with resistance to Kanamycin and protein expression can be induced by Rhamnose.

This thesis focused on the preparation and characterization of these fusion proteins as well as *in vitro* validation and comparison between four types of fusion proteins.

Chapter 2

Materials and Methods

2.1 Fusion Protein Constructs and Plasmid DNA Constructs

pD861-STII vectors containing the fusion protein sequences were synthesized by Atum Bio. Four different fusion protein sequences were included to express as FTB04~07.

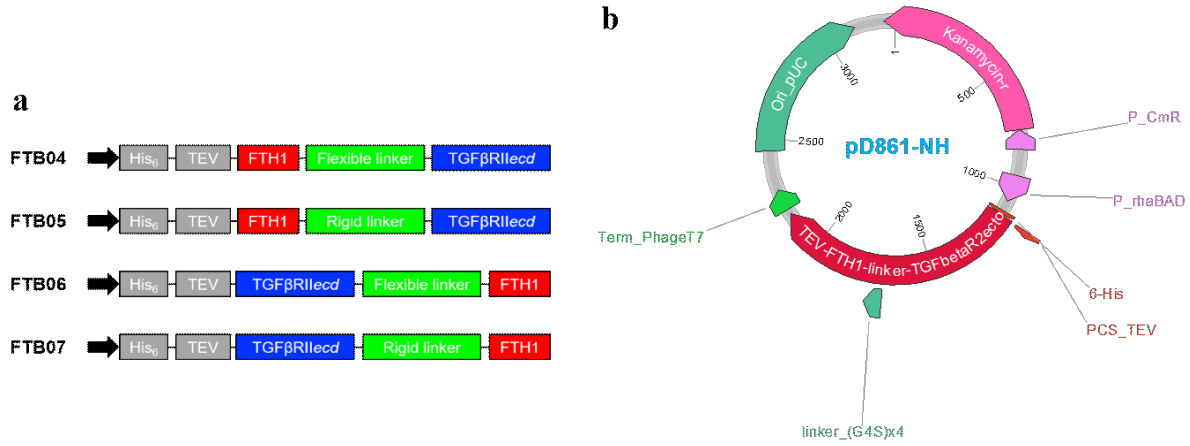

Figure 1. Fusion protein construct and plasmid DNA construct. (a) Fusion protein constructs for four different proteins FTB04~07. (b) An example of plasmid DNA constructs for expression in E. coli system with customized fusion protein sequences. The plasmid DNA sequences included Kanamycin resistance for bacteria selection.

2.2 Bacterial Protein Expression

Invitrogen™ One Shot™ BL21 Star™ (DE3) Chemically Competent E. coli (Thermo Scientific) was transformed with plasmid DNA vectors containing each fusion protein according to the protocol provided. A tube of BL21(DE3) Competent E. coli cells was thawed

on ice for 10 minutes. 0.5 µL of plasmid DNA was added to the cell mixture and mixed by gently flicking the tube. The mixture was placed on ice for 30 minutes. Then the mixture was heat shocked by incubation at 42°C for exactly 30 seconds. The tube was placed on ice for 5 minutes. 250 µL of pre-warmed Invitrogen™ S.O.C. Medium (Thermo Scientific) was added to the tube in sterile environment. The vial was incubated in a shaking incubator at 37°C for 1 hour at 225 rpm. The cell suspension was then spread on LB agar plates containing 50 µg/mL Kanamycin (Quality Biological) in different densities (20 µL for low density and 100 µL for high density).

Single colonies were picked after overnight incubation at 37°C and cultured in 5mL LB broth (Quality Biological) with Kanamycin overnight at 37°C as the starting culture. Then, starting culture was mixed with LB broth with Kanamycin at 1:1000 and incubated at 37°C. Remaining starting culture was preserved at 4°C. When OD600 value reached 0.6, 0.5 mM Rhamnose was added to the E. coli culture and incubated further for 5 hours at 37°C for protein induction. The cells were collected by centrifugation (5000x g, 20 min, 4°C) and washed once with 30 mL DPBS. The pellet was then stored at -20°C overnight. When proper expression was confirmed, remaining starting culture was mixed diluted in 25% glycerol and stored at -80°C as stocks for further usage.

For 500 mL bacteria culture, the cells were lysed with 15 mL B-PER™ Bacterial Protein Extraction Reagent (Thermo Scientific) with 1 mM PMSF and agitated in room temperature for 30 minutes. The pellet was collected by centrifugation at maximum speed for 20 minutes

at 4°C. The pellet was washed once with washing buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl) and once with TBST.

2.3 Protein Refolding

Six different denaturing and refolding conditions were tested in search of the optimal method. High concentration of urea and guanidine-HCl were tested as potential solubilization buffers and refolded with three different redox pair ratios, as shown in Table 1.

Table 1. Refolding conditions tested with FTB04.

1	50 mM Tris-HCl	100 mM Urea	500 mM L-Arg	2 mM GSH	0.2 mM GSSG
2					0.04 mM GSSG
3					0.02 mM GSSG
4		100 mM Gdn-HCl			0.2 mM GSSG
5					0.04 mM GSSG
6					0.02 mM GSSG

The inclusion bodies were solubilized with 20 mL denaturing buffer (50 mM Tris-HCl, 8 M Urea, 5 mM DTT) and rotated at 4°C overnight. The solubilized protein was centrifuged at maximum speed for 15 minutes at 4°C. Supernatant was collected and concentration was measured with BCA assay (Thermo Scientific). The protein was then diluted to 5 mg/mL with denaturing buffer without DTT.

The denatured protein was diluted 10-fold with refolding buffer (50 mM Tris-HCl, 500 mM L-Arginine, pH 8.0, 2 mM GSH and 0.2 mM GSSG) and stirred in 4°C for 3 days. The refolded protein was filtered and re-concentrated with Pierce™ Protein Concentrator PES, 10K MWCO (Thermo Scientific). The concentrated protein was then dialyzed against PBS overnight using

SnakeSkin™ Dialysis Tubing, 10K MWCO (Thermo Scientific).

2.4 Protein Purification

The protein was retrieved from dialysis and incubated with Ni-NTA Agarose (Qiagen) from 5 mL slurry (washed with DPBS) overnight. Flow-through was analyzed with BCA assay to confirm protein binding to the beads. Then the column was washed with 20 mL PBS and eluted with elution buffer (PBS with 500 mM Imidazole). The protein was eluted 1 mL each time and analyzed with BCA assay to confirm the portions with target protein. The target protein was then desalted with PD-10 columns (GE Healthcare) or dialysis against PBS overnight. The purified protein was measured by BCA assay, then aliquoted and stored at -80°C.

2.5 Protein Characterization

Protein expression was confirmed *via* SimplyBlue™ SafeStain (Thermo Scientific) protein staining SDS-PAGE using Novex™ 8-16% Tris-Glycine Mini Gels, WedgeWell™ format (Thermo Scientific). Further confirmation was achieved through western blot against anti-ferritin antibody (Abcam #ab75973). Purified fusion protein was analyzed by GPC using an ÄKTA start chromatography system (GE Healthcare) and compared with H-FTH1 nanocages. Sizes of the fusion proteins were measured by a Nano ZS90 zetasizer (Malvern).

2.6 Canonical TGFβ Signaling Blockade Studies

SMAD2/3 pathway inhibition was demonstrated by HEK-293T SBE-Luc reporter assay. HEK-293T cells (ATCC) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Corning)

with 10% FBS (Gibco™ Fetal Bovine Serum, Thermo Scientific) and 1% p/s. pGL4.48 vector plasmid DNA was diluted in 1mL of Gibco™ Opti-MEM medium (Thermo Scientific). 30 µL of FuGENE HD Reagent (Promega) was added to the DNA solution and incubated for 15 minutes. During incubation period, 20 mL of HEK-293T cell suspension (detached with trypsin) were prepared in complete medium in $2x10^5$ cells/mL. The DNA-lipid complex solution was added to the cell suspension. The cell suspension was then plated at 100 µL/well on white 96-well cell culture plates and incubated overnight.

Complete medium with varying concentration of antagonist (FTB04 to FTB07) was prepared by 3-fold serial dilution with highest concentration of 10 µg/mL and total 9 concentrations each. Then, TGFβ1 (Cell Signaling Technology) was added to the antagonist solutions at final concentration of 5 ng/mL and incubated for 15 minutes. The antagonist-TGFβ1 mixture was treated to the cells at 100 µL/well and incubated overnight. Negative (no TGFβ1, no antagonist) and positive (with TGFβ1, no antagonist) controls were also included. Steady-Glo® Luciferase Reagent (Promega) was used as a substrate and luminescence was measured using microplate reader.

2.7 TGFβ Binding Affinity Studies

TGFβ1 binding affinity was measured in an ELISA method. TGFβ1 was diluted in DPBS (0.5 µg/mL). Corning EIA/RIA high-binding 96-well plates were first coated with diluted TGFβ1 (100 µL/well) and incubated for 24 hours at 4°C. Empty wells were kept for non-specific binding evaluation. The plates were blocked with StartingBlock T20 blocking buffer (200

µL/well, Thermo Scientific) and incubated for 5 minutes at room temperature. The plates were then washed twice with TBST.

Anti-TGFβ1 antibody (Abcam #ab64715) was prepared in varying concentrations in TBST by 4-fold serial dilution with highest concentration of 1 nM and total 12 concentrations each. Diluted antibody was added to the plates and incubated overnight. FTB04 and FTB05 were prepared in varying concentrations in TBST by 4-fold serial dilution with highest concentration of 1 nM and total 12 concentrations each. Diluted fusion protein was added to the plates and incubated overnight. The plates were washed 3 times with TBST and incubated with Anti-Ferritin antibody (dilution factor 1:1000) for 2 hours at room temperature. All the plates were then washed 3 times with TBST. HRP conjugated anti-mouse IgG was diluted in TBST (dilution factor 1:10000), added to the wells at 100 µL/well and incubated for 1.5 h with gentle shaking. The plates were washed 3 times with TBST. 100 µL/well of 1-Step™ Turbo TMB-ELISA Substrate Solution (Thermo Scientific) was added to the wells and incubated for 10 minutes. 100 µL/well of 1N sulfuric acid (Fisher Chemical) was added as stop solution. The plates were read at 450 nm absorbance using a microplate reader.

The binding studies were repeated with TGFβ2 and TGFβ3 proteins (Cell Signaling Technologies) with serial dilution of fusion proteins and anti-TGFβ antibody (CST #3711) starting from 4 nM. The binding affinities were quantified in the same way as described for TGFβ1.

2.8 Phosphorylation Studies with NIH/3T3 cells

Blockade of SMAD-mediated TGFβ receptor signaling pathway was tested through the reduction of SMAD2/3 phosphorylation. NIH/3T3 cells (ATCC) were seeded in 60mm TC treated plates (Fisherbrand) and maintained until 70% confluent. The NIH/3T3 cells were cultured in DMEM with 10% BCS (Bovine Calf Serum, Thermo Scientific) and 1% p/s. Cells were washed and starved for 6 hours with serum-free DMEM. Pre-warmed serum-free medium containing FTB04 and FTB05 (0.1, 1, 10 nM) were prepared and TGFβ1 (5 ng/mL) was added followed by incubation for 15 minutes at room temperature. Cell media were replaced with the fusion protein-TGFβ1 mixture containing media and incubated for 15 minutes. TGFβ1 negative and positive controls were also included. Cells were washed with cold DPBS. Cells were lysed using 100 µL RIPA Lysis and Extraction Buffer (Thermo Scientific) containing Halt™ Protease Inhibitor Cocktail and Halt™ Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific). The lysates were agitated for 30 minutes at 4°C and centrifuged at maximum speed for 15 minutes at 4°C. Supernatant was collected in fresh tubes. Concentration was measured using BCA assay. Samples were loaded 10 µg/well and probed with primary antibodies of anti-pSMAD2 Abs (CST #3108) and anti-pSMAD3 Abs (Abcam #ab52903). Membranes were developed with Pierce™ ECL Western Blotting Substrate (Thermo Scientific), imaged using ChemiDoc™ MP Imaging System (Bio Rad), stripped with Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific) and blocked again with 3% BSA. The membranes were re-probed against SMAD2/3 (CST #8685) and imaged again. The membranes were then stripped and re-blocked again and probed against β-actin (Santa Cruz) to confirm unified

loading conditions.

2.9 Transferrin Receptor 1 Pull-down Assays

Binding affinity with Transferrin Receptor 1 was confirmed by pull-down assay with HepG2 cells. HepG2 cells were cultured in DMEM with 10% FBS and 1% p/s until confluent and lysed with Pierce™ IP Lysis Buffer (Thermo Scientific) with protease inhibitor cocktail. HepG2 lysate was diluted in TBST at final concentration of 0.2 mg/mL. 40 µg of H-FTH, FTB04 and FTB05 were respectively added to the cell lysate and incubated for 1-2 hours at 4°C with gentle agitation. Dynabeads™ His-Tag Isolation and Pulldown (40 µL/tube, Thermo Scientific) were blocked with 1% BSA in 300 µL washing buffer (50 mM phosphate, 300 mM NaCl, 10 mM imidazole, 0.01% Tween20, pH 7.4) for 1 hour. The protein-cell lysate mixture was diluted 5-fold with TBST and transferred to the blocked beads and incubated for 30 minutes at 4°C. The beads were rinsed for four times with 200 µL washing buffer and then eluted by incubation with 30 µL PBS with 250 mM imidazole for 10 minutes at room temperature with agitation. Supernatant was collected in fresh tubes. 10 µL 4x reducing loading buffer was added to the supernatant and heated at 95°C for 10 minutes. Samples were loaded 1 µL/well to be probed against ferritin and 10 µL/well to be probed against transferrin receptor 1 (Abcam #ab214039) in western blot using Novex™ 10-20% Tris-Glycine Mini Gels, WedgeWell™ format (Thermo Scientific).

Chapter 3

Results and Discussion

3.1 Fusion Protein Preparation and Characterization

To engineer recombinant fusion protein with ferritin heavy train and TGFβ receptor 2 ecto domain, we transformed E. coli cells with a custom-designed plasmid DNA encoding specific sequences for the fusion protein with a 6His-tag. Fusion protein with a flexible linker in between was named FTB04 while the one with a rigid linker was named FTB05. Fusion Protein with switched positions of the two functional portions were named FTB06 and FTB07, respectively (Figure 1a). Protein expression was induced with 0.5mM Rhamnose. Fusion protein induction was confirmed by Coomassie Blue stained SDS-PAGE gel and western blot against anti-ferritin antibody.

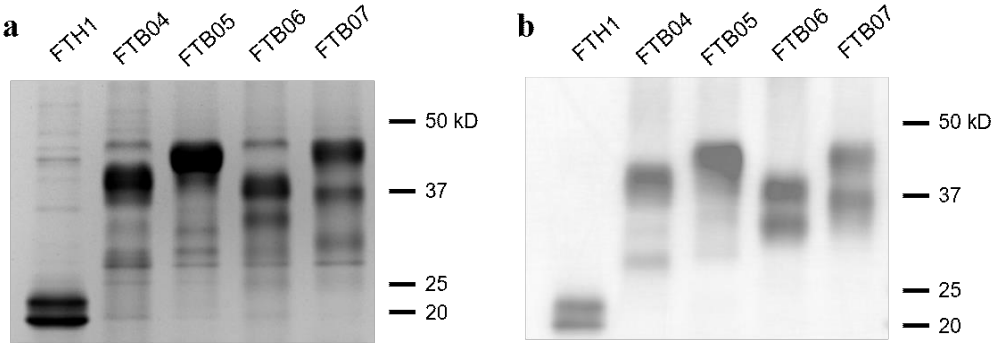

Figure 2. Protein expression was confirmed through SDS-PAGE and western blot. (a) SDS-PAGE protein staining of H-FTH1 and FTB04~07 demonstrated the molecular weight of each type of protein subunits. (b) Western blot of H-FTH1 and FTB04~07 probed against anti-

ferritin antibody further confirmed expression of designed fusion proteins with ferritin heavy chain.

Protein staining and western blot against anti-ferritin antibody confirmed the expression of designed fusion proteins with ferritin H chain. Samples were loaded at 2 μg/well in 8-16% SDS-PAGE gradient gel for SDS-PAGE and 0.1 μg/well in the same gel for western blot. As shown in Figure 2, all fusion proteins were produced with little impurities that do not interact with anti-ferritin antibody. All types of fusion proteins displayed a molecular weight around 40 to 45 kDa as expected.

Inclusion bodies were then solubilized and refolded to present desired nanocage structure. Six different denaturing and refolding conditions (see Table 1) with urea or guanidine-HCl and different ratios of redox pairs were tested. Refolded proteins were compared *via* binding affinity tests with TGFβ1.

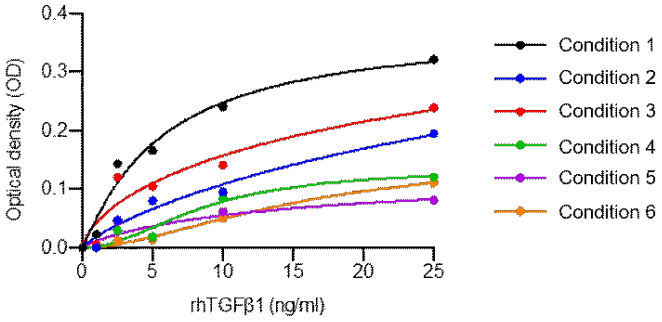

Figure 3. Comparative binding affinity with TGFβ1 of FTB04 under different refolding conditions. FTB04 refolded with six different refolding buffers as listed above were tested to bind with TGFβ1. Binding affinity was measured *via* ELISA and quantified through absorbance at 450nm.

Refolding condition 1, i.e. solubilization with high concentration of urea and refolding with redox pair at 10:1 ratio provided fusion protein with best performance in binding with TGFβ1.

Thus, all types of inclusion bodies were refolded utilizing method 1 for protein preparation and further studies.

Refolded protein was purified using Ni-NTA Agarose resin. Subsequently, fusion protein purification was confirmed by GPC analysis. DLS was measured by a ZS90 zeta-sizer using DPBS as dispersant.

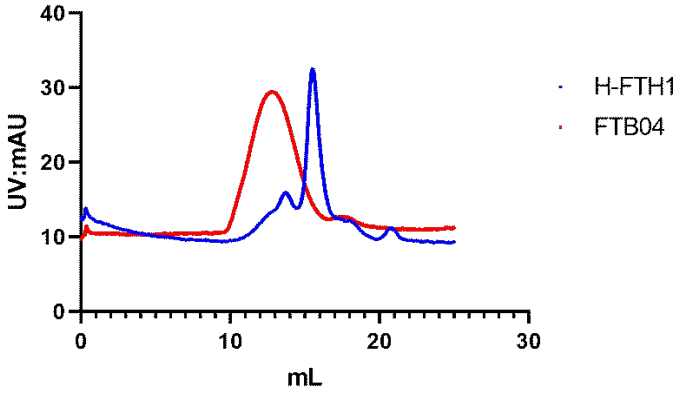

Figure 4. GPC analysis of H-FTH1 and FTB04 after purification. The analysis confirmed the increase in size with inclusion of TGFβRII ectodomain. The curve also confirmed the prepared fusion protein was pure enough for further studies.

Results from the GPC analysis confirmed that Ni-NTA purification provided fusion protein FTB04 with barely any impurities that can be used for further validation studies. The shift of the peaks indicated the fusion protein is larger than H-FTH1 in size.

Table 2. Size measurements of FTB04 to FTB07 compared with H-FTH1.

Fusion Protein	Z-Average Hydro-diameter (nm)	PDI
H-FTH1	15.45±0.50	0.197
FTB04	28.16±0.20	0.187
FTB05	31.90±0.50	0.172
FTB06	19.57±0.20	0.167
FTB07	29.13±0.10	0.174

Size measurements confirmed the difference between fusion proteins and FTH nanocages. Similar PDI levels indicated that the fusion proteins formed proper structure similar to H-FTH1 nanocages after refolding. The size measurement results are consistent with the molecular weights (shown in Figure 2). Sequences with rigid GS linkers produced larger particles. As all types of fusion proteins are refolded into proper nanoparticles, we proceeded to *in vitro* validations with all four types of fusion proteins.

3.2 Blockade of TGFβ Signaling Pathway by Fusion Protein

To evaluate whether these TGFβ traps can block SMAD-mediated TGFβ signaling pathway as shown in other previous studies, HEK-293T cells transfected with SBE-luciferase gene were treated with a mixture of fusion protein and TGFβ1. Luminescence of activated luciferase demonstrated SMAD pathway signaling.

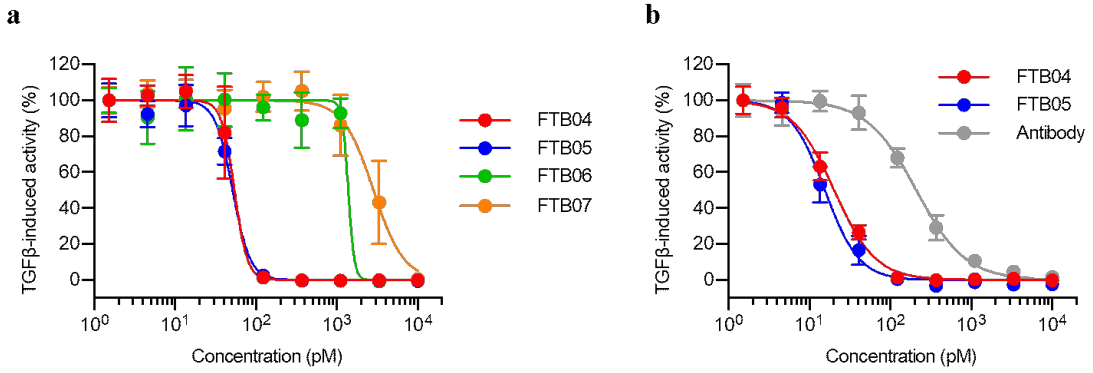

Figure 5. TGFβ signaling pathway blockade by fusion proteins in comparison with commercial antibody. (a) The abilities of FTB04~07 in blocking TGFβ signaling pathway were compared through HEK-293T SBE-luciferase reporter assay. (b) FTB04 and FTB05 were further compared with commercial anti-TGFβ antibody through the same assay.

Results from the HEK-293T SBE-luciferase reporter assay indicated that FTB04 and FTB05

presented superior ability to block the TGFβ signaling pathway compared with FTB06 and FTB07. While FTB06 and FTB07 are capable of blocking the TGFβ induced activity starting from a concentration between 10^3 to 10^4 pM, FTB04 and FTB05 demonstrated strong blockade at a concentration as low as 10^2 pM. Therefore, we proceeded further studies with only FTB04 and FTB05.

Further comparison as shown in Figure 5 (b) indicated that FTB04 and FTB05 possessed blocking ability even better than commercial anti-TGFβ1 antibody. Commercial anti-TGFβ1 antibody can mostly block TGFβ induced activity at around 10^3 pM. The difference in the abilities to block the TGFβ signaling pathway is assumed to be due to the difference in protein structures between the fusion protein and the traditional antibody TGFβ traps. One of the greatest advantages of ferritin H chain as nanocarriers is the cage structure these subunits fold into. The nanocages with multi-valency can lead to higher interactive activities. In this case, the multi-valent FTH nanocages result in higher efficiency in blocking TGFβ induced activities. As the TGFβ blocking function met our expectation, further studies were performed to confirm the functional mechanism of the fusion protein TGFβ traps.

3.3 Fusion Protein Traps TGFβ

To evaluate the binding affinity of the fusion proteins with TGFβ, i.e. the ability of the fusion proteins to trap TGFβ, Enzyme-linked immunosorbent assay (ELISA) was performed to quantify the binding affinity. Varying concentrations of fusion proteins were prepared by serial dilution starting from 1 nM and treated to TGFβ1 coated plates. The wells were incubated with

ferritin antibody followed by HRP conjugated secondary antibodies. Turbo TMB substrate was added to quantify the binding affinity. The amount of fusion protein bound were measured through absorbance at 450 nm and compared with commercial Anti-TGFβ antibody.

The same assay was repeated with TGFβ2 and TGFβ3 with higher concentrations of antagonists as the binding affinity differs. In binding studies with TGFβ2/3, both fusion proteins and antibody were included *via* serial dilution starting from 4 nM. The quantified binding affinity was measured in the same way.

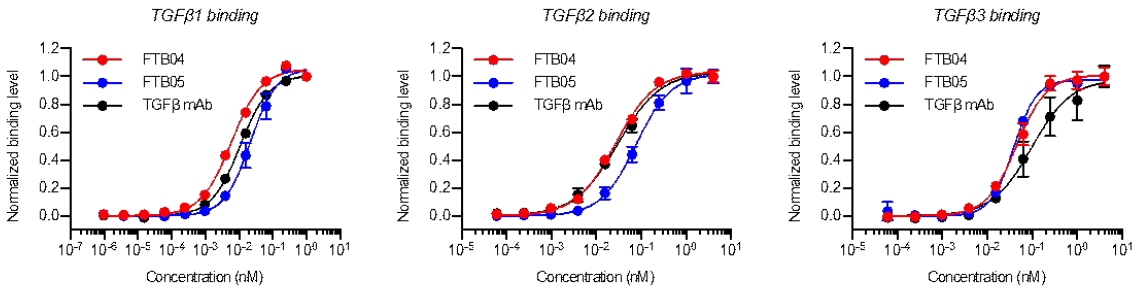

Figure 6. TGFβ binding affinity with FTB04 and FTB05 in comparison with commercial anti-TGFβ antibody. All three types of TGFβ were tested individually.

As shown in Figure 6, both fusion proteins possessed ability to trap all three types of TGFβ almost identical to that of commercial anti-TGFβ antibody. All antagonists tested achieved almost full binding level at around 1 nM. Among them, FTB04 demonstrated superior binding affinity with all three types with TGFβ proteins. FTB04 trapped almost all TGFβ1 at around 10^{-1} nM, four fold lower than FTB05 or the antibody. For TGFβ2, commercial antibody demonstrated comparable binding activities compared to FTB04. For TGFβ3, both fusion protein displayed similar binding affinity, slightly higher than that of the antibody. In conclusion, FTB04 presented the best performance in trapping the TGFβ proteins while FTB05

and commercial anti-TGFβ antibodies also presented decent binding affinity with the proteins.

While binding affinity against TGFβ proteins is similar among fusion proteins and commercial antibodies, blockade of TGFβ-induced activity is more efficient with FTB04 and FTB05 (Figure 5b). One possible reason is the difference in binding conditions in two studies. In the binding affinity studies, TGFβ proteins were immobilized on protein binding plates. The binding sites available for the antagonists were consistent and cannot be affected by the multi-valency of the nanocages. In the blockade studies, TGFβ1 was dispersed in the media and can be captured by all 24 subunits of the nanocages at equal chances, while each molecule of TGFβ antibody only possessed one binding site. Thus, the blockade ability represents the avidity of the antagonists instead of single interaction affinity. The different conditions of TGFβ proteins resulted in the difference in the two studies.

3.4 Fusion Protein blocks SMAD-Mediated TGFβ Signaling Pathway

We have previously confirmed that fusion protein FTB04 and FTB05 are capable of binding to TGFβ proteins and blocking the TGFβ signaling pathway *via* HEK-293T SBE-luciferase reporter assay. To determine whether fusion protein blocked the SMAD-mediated TGFβ signaling pathway by inhibiting the phosphorylation of SMAD2 and SMAD3 transcription factors as designed in our theory, further phosphorylation studies were performed with mouse fibroblast NIH/3T3 cells, which has been characterized to be readily responsive to SMAD-mediated TGFβ signaling[48].

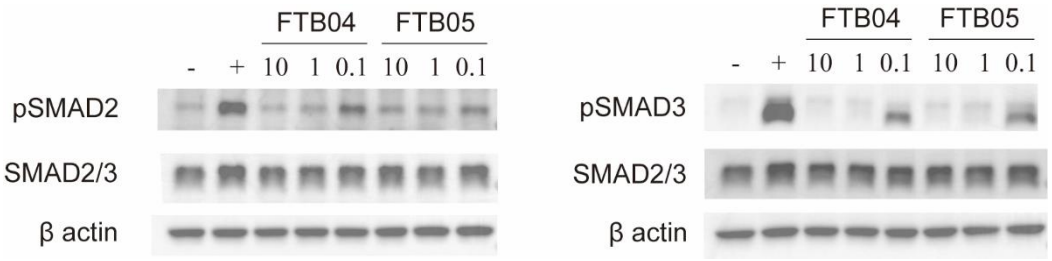

Figure 7. Phosphorylation studies of SMAD2 and SMAD3 with FTB04 and FTB05. Serum-free medium with neither TGFβ1 nor fusion protein was included as negative control. Medium with only TGFβ1 was included as positive control. Fusion protein FTB04 and FTB05 were treated at three different concentrations to compare their effects on the blockade of SMAD phosphorylation.

Samples were loaded 10 μg/well in 8-16% SDS-PAGE gradient gel. In each figure, samples were loaded in the order (from left to right) of: negative control, positive control, TGFβ1 with 10, 1, 0.1 nM of FTB04 followed by FTB05.

As presented in the positive control, presence of TGFβ1 led to high level of phosphorylation of both SMAD2 and SMAD3 in NIH/3T3 cells. Figure 7 clearly demonstrated that at a concentration as low as 1 nM, both FTB04 and FTB05 can completely block the phosphorylation of SMAD2 and SMAD3. The phosphorylation studies confirmed that fusion protein FTB04 and FTB05 possess the ability to block the phosphorylation of SMAD2/3 proteins, thus block the SMAD-mediated TGFβ signaling pathway.

3.5 Fusion Protein Binds to Human Transferrin Receptor 1

To determine whether our fusion protein possess the ability to traverse the blood brain barrier (BBB) by transcytosis mediated by Transferrin Receptor 1 (TfR1), we performed pull-down assay with human liver cancer HepG2 cells. HepG2 cell lysate was incubated with fusion protein and then pulled down by His-tag binding magnetic beads.

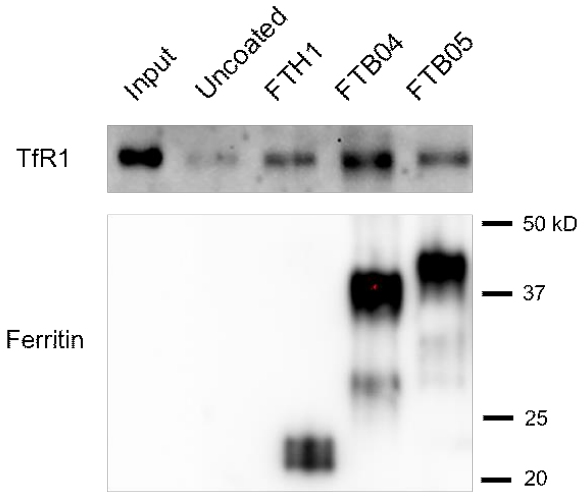

Figure 8. TfR1 Pull-down assay with fusion proteins and comparison with H-FTH1. H-FTH1 and FTB04 and FTB05 were incubated with same amount of HepG2 cell lysates and pulled down by magnetic beads. HepG2 cell lysate input was included as positive control. Uncoated magnetic beads without protein-lysate mixture incubation was included as negative control.

Samples were loaded (from left to right) in the order of: HepG2 cell input, uncoated negative control, H-FTH, FTB04, FTB05. 0.2 µg of HepG2 cell lysate input was loaded as positive control. Eluate from magnetic beads without protein bound was loaded as negative control. 20 µg of HepG2 cell lysates were mixed and incubated with 40 µg of each type of fusion proteins. As shown in Figure 8, TfR1 is expressed abundantly in HepG2 cells and barely binds to BSA-blocked magnetic beads. Fusion proteins are confirmed to be able to bind to human TfR1 while FTB04 possessed improved binding affinity compared to FTB05. The specific binding against TfR1 provided the fusion proteins with potential to traverse human blood brain barrier for treatment of glioblastoma.

Chapter 4

Conclusions

In this project, we engineered fusion proteins with the potential of traversing human blood brain barrier and trapping TGFβ for treatment of glioblastoma in a combination with immune checkpoint inhibitors. Fusion proteins incorporating ferritin H chain and TGFβ receptor 2 ectodomain connected *via* a flexible or rigid GS linker were designed as FTB04 to FTB07 with switched positions. All fusion proteins were expressed through BL21(DE3) *E. coli* system and expressed as insoluble inclusion bodies. Six different solubilization and refolding buffers were tested and we determined the optimal solubilization and refolding condition was denaturing with high concentration of urea and refolding with 10:1 GSH-GSSG redox pair. The inclusion bodies were solubilized, refolded and purified by Ni-NTA agarose resin. The purified protein was characterized through GPC analysis and size measurements. Characterization confirmed the inclusion bodies were refolded into fusion proteins with decent purity and size. Then we proceeded to *in vitro* validations and comparison of the four types of fusion proteins.

HEK-293T SBE-luciferase reporter assay was initially performed to validate whether the fusion proteins were capable of trapping TGFβ and blocking TGFβ signaling pathway. Our result indicated that FTB04 and FTB05 possessed superior ability in blocking the TGFβ signaling pathway compared to FTB06, FTB07 as well as commercial anti-TGFβ antibody.

Thus, we proceeded to further assessments without FTB06 and FTB07. Studies of the binding affinities between fusion proteins and TGFβ proteins TGFβ1~3 were carried out through ELISA. The result indicated that both fusion proteins presented similar binding affinity with all three types of TGFβ proteins as commercial TGFβ antibody. Among the antagonists involved, FTB04 demonstrated the strongest binding affinity compared to the others. Phosphorylation Studies with NIH/3T3 cells indicated that the presence of FTB04 and FTB05 at a concentration as low as 1 nM blocked the phosphorylation of SMAD2 and SMAD3 proteins, confirming the designing theory of the fusion proteins blocking the SMAD-mediated TGFβ signaling pathway. Pull down assay with transferrin receptor 1 expressing HepG2 cells was performed with His-tag affinitive magnetic beads. The result confirmed TfR1 as the receptor for the fusion proteins. FTB04 showed similar binding with TfR1 as H-FTH1, stronger than that of FTB05.

In conclusion, the designed fusion proteins can be successfully expressed through E. coli system and be refolded into nanocages with decent purity. *In vitro* validations confirmed that our fusion protein could trap TGFβ proteins and block the TGFβ signaling pathway by blocking phosphorylation of SMAD2 and SMAD3 proteins. Binding studies with TfR1 confirmed that the fusion proteins could interact with human TfR1, thus possess the potential for receptor-mediated transcytosis across human blood brain barrier. All assessment results supported that FTB04, with TGFβRII ectodomain on the N terminus and a flexible linker, demonstrated the best performance in binding with TfR1 and blocking SMAD-mediated TGFβ signaling pathway. For further studies, we will proceed with only FTB04. We would validate the *in vitro*

penetration of human blood brain barrier with human BBB ECs. Furthermore, we could proceed to *in vivo* studies with GL261-Luc2 glioblastoma syngeneic orthotopic model and assess the anti-tumor activity in combination with anti-PD-L1 therapy.

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Education Background

Johns Hopkins University	Chemical and Biomolecular Engineering *Master of Science in Engineering* GPA:3.87/4.0	*2017.08~2019.05*
South China Normal University	Environmental Engineering *Bachelor of Engineering* GPA:3.61/5.0 Ranking:3/60 Chinese Language and Literature (Minor)	*2013.09~2017.06* *2014.07~2017.06*

Research Experience

Graduate Research Center for Nanomedicine *PI: Dr. Jung Soo SUK*

BBB Penetrating TGFβ Trap for Treatment of Glioblastoma with a Combination of Immune Checkpoint Inhibitors *2018.05~Now*

- Optimize E. coli culture conditions for designed protein expression
- Fusion protein induction, extraction, purification and protocol optimization
- Fusion protein validation (ELISA etc.)
- *In vitro* cell studies, phosphorylation studies etc.
- *In vivo* drug distribution studies
- *In vivo* study with a combination with anti-PD-1/PD-L1 therapy

Bio-reducible Polymer Based Gene Therapy *2017.09~2018.05*

- Bio-reducible PEI synthesis and optimization
- Nanoparticle surface modification by PEGylation and particle characterization
- *In vitro* cytotoxicity and efficiency study
- *In vivo* particle distribution and efficiency studies

Focused Ultrasound (FUS) Mediated Drug Delivery in Bladder *2017.11~Now*

(Collaboration)

- PEGylated and non-PEGylated fluorescent nanoparticles preparation
- FUS mediated particle *in vivo* distribution studies with MRI
- Rat bladder tissue sectioning and imaging

Undergraduate Dissertation *PI: Dr. Feng JIANG*

Effectiveness of Alkali Method for Odor Gases Control in Sewage System *2016.09~2017.05*

- Sulfate-reducing bacteria (SRB) culture and acclimation
- Set up odor gases emissions mathematical model
- Design and conduct simulation experiment of the sewage system and verify

mathematical model

- Offer advices on the Hong Kong HATS Project based on the model

Student Innovation and Entrepreneurship Training Program *PI: Dr. Bingyan LAN*

Mechanism of Lead Adsorption by Weak Crystalline Iron Oxide *2015.06~2017.04*

- Design and optimize ferrate synthesis scheme
- Prepare high purity ferrate and ferrihydrite adsorbent and characterize the products
- Quantify adsorption efficiency by applying products to heavy metal ions in solution

Awards and Competitions

MSE 2nd year scholarship	Department	*2018~2019*
Excellent Interns of School of Chemistry and Environment	College-wide	*2016~2017*
Second-class Merit Scholarship	University-wide	*2015~2016*
First-class Merit Scholarship	University-wide	*2014~2015*
Excellent Student Cadre in School of Chemistry and Environment	College-wide	*2013~2014*

Publications

X Huang*, J Zhuang*, SW Chung*, B Huang, G Halpert, K Negron, X Sun, **J Yang**, Y Oh, P M Hwang, J Hanes, JS Suk *"Hypoxia-tropic Protein Nanocages for Modulation of Tumor- and Chemotherapy-associated Hypoxia"*	ACS Nano	*Dec.21 2018*

Internship

South China Sea Institute of Oceanology, Chinese Academy of Sciences *2016.07~2016.12*

Research Assistant, Marine Environmental Engineering Center

- Training in detecting and monitoring seawater and marine sediments properties
- Analyze the quality of seawater and marine sediments in the South China Region, mainly worked on the detection and analysis of nutrients, DO, BOD, COD, TOC and sulfide